Reference: *Biol. Bull.* **169**: 256–266. (August, 1985)

A NOVEL FMRFamide-RELATED PEPTIDE IN *HELIX*: pQDPFLRFamide

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Abstract

A novel FMRFamide-like peptide, purified from the ganglia of *Helix aspersa*, has the amino acid sequence: pyroglutamyl-aspartyl-prolyl-phenylalanyl-leucyl-arginyl-phenylalanine amide (pQDPFLRFamide). Synthetic pQDPFLRFamide was prepared; it is chromatographically and biologically indistinguishable from the natural peptide, confirming the sequence. pQDPFLRFamide is about a hundred times more potent than FMRFamide on the isolated *Helix* heart, but slightly less potent than FMRFamide on the *Busycon* radula protractor muscle. Since pQDPFLRFamide occurs in *Helix* blood at levels sufficient to excite the isolated *Helix* heart, it may act as a cardioregulatory hormone.

Introduction

The molluscan neuropeptide FMRFamide (phenylalanyl-methionyl-arginyl-phenylalanine amide) was originally isolated from the ganglia of a clam, *Macrocallista nimbosa* (Price and Greenberg, 1977a), but its occurrence is not restricted to this species, nor even to clams in general. Indeed, the ganglia of all molluscan species examined contain FMRFamide-like biological activity (Agarwal *et al.*, 1972). Moreover, FMRFamide itself appears to be ubiquitous in molluscs and, in some species [*e.g., Aplysia brasiliana* (Lehman *et al.*, 1984)], to account for all the FMRFamide-like activity present. In some gastropods, however, related peptides also occur and may even be the predominant forms (reviewed in Price, 1986).

Pulmonate ganglia, in particular, have activity not attributable to FMRFamide. In *Helix aspersa*, for example, the ganglia contain, not only FMRFamide, but also related peptides distinguishable by their chromatographic behavior and biological activity (Cottrell *et al.*, 1981; Price, 1982). Cottrell *et al.* (1981) found that partially purified FMRFamide from the ganglia of *Helix aspersa* has much more excitatory activity on the *Helix* heart than could be accounted for by FMRFamide itself, and Greenberg and Price (1980) used the heart of *Helix* and the radula protractor muscle of *Busycon* as a parallel bioassay to calculate this excess at about 600-fold.

Here we describe the purification of one of these peptides and the determination of its amino acid sequence as: pyroglutamyl-aspartyl-prolyl-phenylalanyl-leucyl-arginyl-phenylalanine amide (pQDPFLRFamide). We show that, although pQDPFLRFamide is somewhat less potent than FMRFamide on the radula protractor, it is about 100 times more potent than FMRFamide on the heart of *Helix*. Thus this heptapeptide can account for most of the excess cardioexcitatory activity found in *Helix* brain. Furthermore, we show that the blood levels of pQDPFLRFamide in *Helix* are sufficient to affect the heart, so it may be a cardioregulatory hormone.

Received 21 January 1985; accepted 16 May 1985.

Materials and Methods

Animals

Helix aspersa were collected in southern California and Scotland. Whelks (*Busycon contrarium*) were collected from the Gulf of Mexico south of Tallahassee, shipped to the Whitney Laboratory, and maintained there in natural seawater (31‰) at 23°C. Clams (*Mercenaria mercenaria*) were readily obtained from the inland waters adjacent to Marineland, Florida.

Extraction

Circumesophageal ganglia (800 in all, about 15 g wet weight) were crudely dissected from large snails, and each was immediately put in acetone (100 ml total). The ganglia in acetone were left in the freezer until all of the tissue had been collected. The acetone was then poured off, centrifuged at 25,000 × *g* for 10 min and the supernatant saved. The ganglia were homogenized in 80% acetone (50 ml) and centrifuged (10,000 × *g* for 10 min); the pellet was extracted again, and the supernatants were combined with the original acetone. The combined acetone extracts were evaporated under reduced pressure (water aspirator) in a rotary evaporator with its bath temperature gradually being increased from room temperature. At 40°C, the water started to distill, and the remaining liquid (mainly water) was centrifuged again; the supernatant was forced through a disposable C-18 cartridge (Waters Sep-Pak). The retained material was eluted with methanol (5 ml), and this methanol was then evaporated.

Purification

The residue was taken up in 0.1 *M* acetic acid (3 ml), applied to a column of Sephadex G-15, and eluted with the same. Those fractions containing FMRFamide were detected by a radioimmunoassay (RIA) described below. The peak of immunoreactivity was centered at fraction 21 (Fig. 1A); its component fractions were combined and lyophilized. The residue was again taken up in 0.1 *M* acetic acid and run through the Sephadex column, and the immunoreactive fractions were pooled and lyophilized. A portion of this material was reserved for tryptic digestion (see below). The remainder was taken up in buffer (0.2 ml of 0.5 *M* ammonium acetate with 0.1 *M* acetate acid, pH 5.5, in n-butanol to 80% of saturation), injected onto a C-18 reserve phase column (Waters Radial-Pak), and eluted with the same solvent at 4 ml/min (Fig. 1B). Fractions 30–34 were lyophilized, redissolved in 0.5 ml of 50 m*M* ammonium acetate (pH 6.0), and applied to a small column of CM-Sephadex (0.35 × 25 cm; V_T = 2.2 ml). The columns was eluted with 10 ml of 50 m*M* ammonium acetate (pH 6.0) and then with a gradient to 500 m*M* ammonium acetate (40 ml total volume) as previously described (Price and Greenberg, 1977b) (Fig. 1C).

Characterization

An aliquot of the partially purified (Sephadex G-15) peptide, containing about half a nanomole of immunoreactivity, was digested with trypsin (10 μg) for 3 h at room temperature (pH 8.0). The material was lyophilized, then dansylated and chromatographed on polyamide TLC according to the method of Tatemoto and Mutt (1978) for amino acid alpha-amides.

An aliquot of the fully purified peptide was hydrolyzed in redistilled, constant boiling hydrochloric acid, at 108°C, for 16 h, in a sealed glass tube under nitrogen. The amino acids were determined with an automatic analyzer (Hitachi 835).

Further aliquots of purified peptide were subjected to digestion with carboxypeptidase Y (10 μg per 50 μl) at room temperature, for 20 or 90 min. Digestion was stopped by dilution with the amino acid analyzer sample-buffer (0.02 *M* HCl), and was followed by immediate injection onto the column of the analyzer.

Paper electrophoresis was done on Whatman No. 1 paper with 1% triethylamine as the buffer and a potential of 15V/cm for 70 min. The natural peptide was located by cutting the paper into sections, immersing each in RIA buffer overnight in the refrigerator, and finally taking aliquots for RIA. The peptides used as standards were synthesized by Peninsula Laboratories (San Carlos, California) and were detected on the paper strips by the Sakaguchi (Stahl, 1962) test for arginine.

Synthesis

Two peptides, pQDPFLRFamide and its 2-asparaginyl analog (pQNPFLRFamide), were synthesized on 4-methyl-benzhydrylamine resin by the solid phase method. The peptides were released from the resin and deprotected with anhydrous hydrogen fluoride. They were purified in two steps: chromatography on Sephadex G-25 with 0.05 *M* ammonium bicarbonate as elutant, followed by partition chromatography on Sephadex G-25 with n-butanol/water/acetic acid (4:1:5) as the solvent. Purity was checked by amino acid analysis and by thin-layer chromatography on silica gel [solvent: n-butanol/water/acetic acid/pyridine (15:12:3:10)], developed with both the Sakaguchi and chlorine-iodide reagents.

Radioimmunoassay (RIA)

A rabbit antiserum to a conjugate of YGGFMRFamide and thyroglobulin was used in the RIA, and iodinated YGGFMRFamide was the trace; the preparation of antiserum and trace has been described (Price, 1982). The assay was performed as follows. An aliquot (2 μl) of each fraction was transferred to a glass test tube with an automatic diluter (Micromedic model 30010) that added RIA buffer (48 μl) to give a sample volume of 50 μl. The RIA buffer contains 0.01 *M* sodium phosphate with 1% bovine serum albumin, 0.9% sodium chloride, 0.01% merthiolate, and 0.025 *M* sodium EDTA adjusted to pH 7.0; a buffer without the albumin was used for sample dilution when even traces of protein had to be prevented from contaminating the fractions. The trace (in 100 μl of buffer) was added together with diluted antiserum (also 100 μl) to each tube; the final dilution of antiserum was about 1:60,000, and there were 10,000 CPM of trace. All of the tubes were then left overnight in the cold (4°C), and 1 ml charcoal solution was added in the morning. (Charcoal solution contains 0.25% charcoal, 0.025% dextran, and 0.01% merthiolate in 0.1 *M* sodium phosphate, pH 7.5; it is stirred overnight before the first use and kept in the refrigerator thereafter.) The charcoal was centrifuged down after 15 min (2500 × *g* for 15 min), and the supernatants were decanted and counted.

Bioassays

FMRFamide-like biological activity was assayed with the radula protractor muscle of *Busycon contrarium,* and the hearts of *Mercenaria mercenaria* and *Helix aspersa.* The use of the radula protractor and clam heart bioassays were described by Price and Greenberg (1977), and that of the *Helix* heart by Kerkut and Cottrell (1963).

Chromatographic comparisons

The synthetic and natural peptides were compared in several HPLC systems and on TLC. In addition to the HPLC system used for the purification (see above), two

gradient elution systems were employed with a u-Bondapak C-18 column: 0.02 *M* phosphate buffer (pH 6.5) with acetonitrile grading from 25% to 45%; and 0.2% trifluoracetic acid with methanol from 30% to 50%. TLC was done on Eastman cellulose layers with n-butanol/acetic acid/water (4:1:2) as the solvent.

Blood determinations

One by one, snails were shelled, their aortas were cut, and the flowing blood was drawn into a calibrated pipet and quickly added to an at least 10-fold excess of acetone in a microfuge tube. Typically, an aliquot of 0.1 ml was added to 1.0 ml of acetone. The tubes were stored in the freezer for several hours and spun in a Beckman Microfuge for 3 min; the supernatants were transferred to clean tubes and dried under streams of air. Finally, the residue was dissolved in RIA buffer, and successive two-fold dilutions were assayed.

Pooled blood from 75 snails was prepared similarly, except that the blood from each snail was not measured individually, but was added to a common container of acetone. The volume of blood collected was determined by the increase in total volume. After the mixture had chilled overnight, it was centrifuged at 10,000 × *g* for 10 min; the supernatant was decanted and dried in a rotary evaporator. The residue was taken up in 0.5 ml of HPLC buffer and injected onto a Waters C-18 Bondapak column which was eluted with a gradient of acetonitrile in 0.7% trifluoroacetic acid at the rate of 2 ml/min. Fractions of 0.5 ml were collected and aliquots subjected to radioimmunoassay. Synthetic peptides were run to confirm the elution times, but only after the blood extract had been chromatographed.

RESULTS

Purification

FMRFamide-like immunoreactivity eluted from the gel-filtration (G-15) column at about three void volumes (Fig. 1A), the usual position (Price, 1986). An aliquot of this peak was subjected to trypsin digestion followed by dansylation and chromatography, and phenylalanine amide was the only amino acid alpha-amide present. Thus the material was already somewhat purified by this one step.

Several peaks of FMRFamide-like immunoreactivity eluted from HPLC (Fig. 1B). The earliest of these (at fraction 9 in Fig. 1B) co-elutes with oxidized FMRFamide, and the next one (at fraction 12 in Fig. 1B) co-elutes with FRMFamide itself. Moreover, if an acetone extract of ganglia is oxidized with either Chloramine T or hydrogen peroxide before HPLC, the peak corresponding to FMRFamide is no longer detected, the peak co-eluting with oxidized FMRFamide is augmented, and the remaining peaks are not affected (not shown). This observation supports the identification of the first two peaks and also implies that the remaining ones do not contain methionine.

The FMRFamide-like immunoreactivity eluted from CM-Sephadex just after the void volume (Fig. 1C), the same elution position as observed for the biological activity in preliminary experiments (Cottrell *et al.*, 1981).

Characterization

An amino acid analysis of the purified peptide (*i.e.*, fraction 3 from CM-Sephadex; Fig. 1C) was in substantial agreement with a corrected, earlier analysis (Price, 1982) and indicated the following composition: Arg_1, Asp_1, Glu_1, Leu_1, Phe_2, Pro_1 (Table I).

Digestion with carboxypeptidase Y led to the rapid appearance of phenylalanine, arginine, and leucine in the reaction mixture, and even by 20 min phenylalanine was

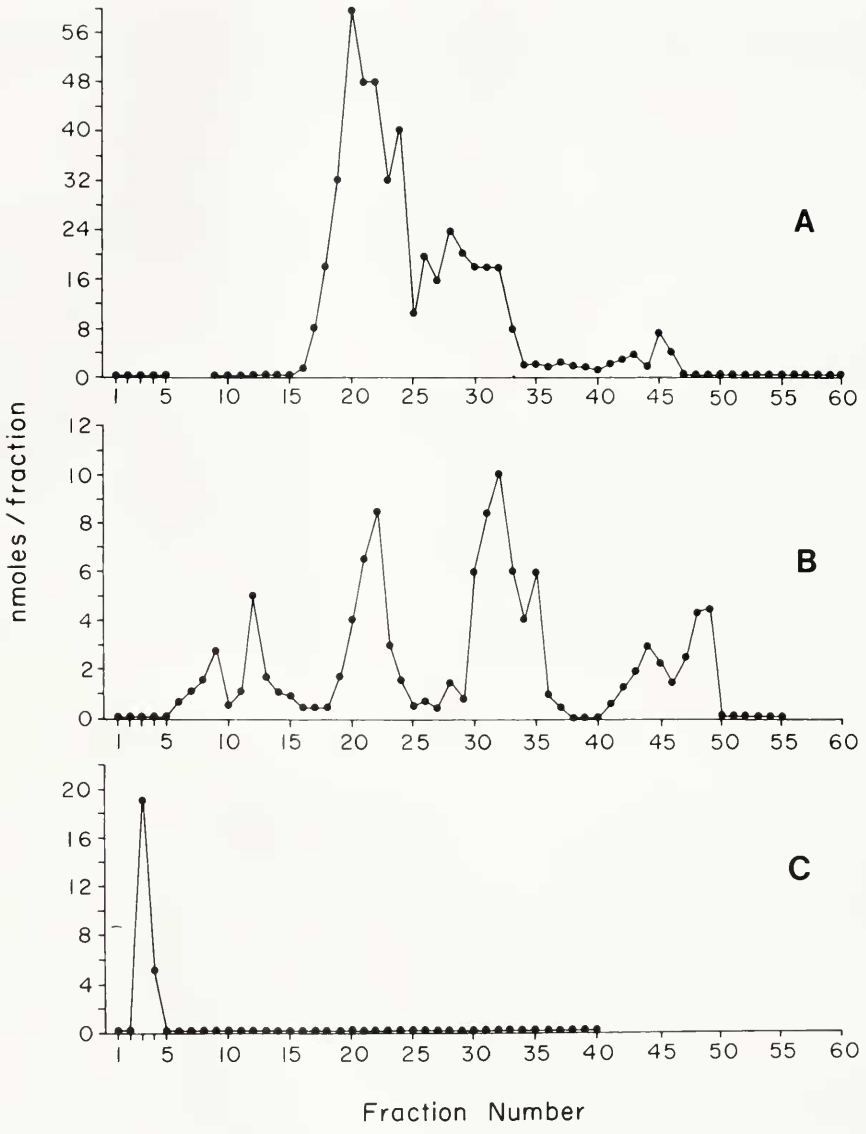

FIGURE 1. Purification of the *Helix* FMRFamide-like peptide. Crudely dissected ganglia were extracted in acetone; the supernatant was dried; the residue was dissolved in 0.1 *M* acetic acid, run through a C-18 Sep-Pak cartridge, and the adsorbed peptides were eluted with methanol (details in Materials and Methods). (A) The methanol was evaporated, and the residue was taken up in 0.1 *M* acetic acid and applied to a Sephadex G-15 column (void volume = 65 ml). Fractions of 8 ml were collected, and aliquots (2 μl) of each fraction were taken for RIA. Fractions 16–25 (the peak of activity) were pooled and lyophilized. (B) The residue was taken up in HPLC buffer and run under the conditions described in Materials and Methods. (C) Fractions 30–34 were combined, lyophilized, dissolved in a small volume (0.5 ml) of 50 m*M* ammonium acetate (pH 6.0) and applied to a CM-Sephadex column eluted with the same solvent. After collecting ten fractions of .5 ml and 5 fractions of 1.0 ml, a gradient to 500 m*M* ammonium acetate (pH 7.0) was started. Twenty fractions of 2 ml each were collected and finally pure 500 m*M* ammonium acetate was used as elutant for the last 5 fractions. The active material was completely nonretained and was recovered, almost entirely, in the third 0.5 ml fraction. In each graph, the immunoreactivity shown is the total in the fraction based on FMRFamide as standard.

TABLE I

Amino acid composition of a Helix *FMRFamide-like peptide*

Amino acid	Quantity (nmole)		Ratio to Leu		Presumed # of residues
	(1)	(2)	(1)	(2)	
Arginine	4.40	10.3	0.99	0.99	1
Aspartic acid	4.70	11.8	1.06	1.44	1
Glycine	0.67	6.0	0.15	0.58	0
Glutamic acid	4.43	10.5	1.00	1.01	1
Isoleucine	0	1.5	0	0.14	0
Leucine	4.43	10.4	1.00	1.00	1
Phenylalanine	8.88	26.1	2.00	2.51	2
Serine	0.24	3.4	.05	0.35	0
Ammonia	6.25	n.d.	1.42	—	1 or 2
Proline	4.46	10	1.01	1	1

The purified peptides, in sealed ampules under nitrogen, were hydrolyzed in constant boiling hydrochloric acid, at 108–110°C, for 16–20 hours. Data set (1) was obtained from a peptide preparation purified as described in the section on Materials and Methods and analyzed on a sensitive amino acid analyzer (Hitachi 835-50). Data set (2) was obtained from a peptide purified by two successive isocratic reverse-phase HPLC runs from an 80% aqueous acetone extract of *Helix* ganglia and analyzed on a relatively insensitive amino acid analyzer (JEOL 6-AH). The first report of this analysis (Price, 1982) contained two errors corrected here: proline, undetected by the integrator was not reported (the value above was determined by manual integration of the record); and the values for leucine and isoleucine were inadvertently transposed. Ammonia in data set (2) could not be determined because of high background levels.

present at 1.5 times the level of leucine. After 1.5 h of digestion, free proline was present at about two-thirds the level of leucine, and phenylalanine at nearly twice the level of leucine but aspartic acid, asparagine, glutamic acid, or glutamine were not detectable. These findings, together with the previously reported ninhydrin negativity (Price, 1982) and the electrophoretic mobility reported below, allow us to deduce the sequence of this peptide, as follows.

The release of phenylalanine amide by trypsin means that the C-terminal dipeptide sequence must be -Arg-Phe-NH_2 because trypsin cleaves on the carboxyl side of basic amino acid residues, and the amide can only arise from the C-terminal amino acid. The results of carboxypeptidase Y digestion show that the last four amino acids are two phenylalanines, arginine and leucine. Moreover, leucine must occur between the phenylalanines, since the Phe/Leu ratio increases from 20 to 90 min. Thus, we must have -Phe-Leu-Arg-Phe-NH_2. Proline becomes detectable very slowly and must therefore follow the second Phe residue; hence, -Pro-Phe-Leu-Arg-Phe-NH_2. The ninhydrin-negativity means that the N-terminal must lack a free amino group, suggesting a pyroglutamyl residue and the partial sequence: pGlu-Asx-Pro-Phe-Leu-Arg-Phe-NH_2.

The identity of the Asx residue was established by paper electrophoresis. The electrophoretic conditions were chosen so that the two model peptides—pGlu-Asp-Phe-Ile-Arg-Phe-NH_2 and pGlu-Asn-Phe-Ile-Arg-Phe-NH_2—ran in opposite directions. When the *Helix* peptide and the model peptides were run in parallel, the *Helix* peptide, like the Asp^2-standard, migrated toward the anode. Thus the sequence of the *Helix* peptide must be: pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (pQDPFLRFamide expressed in the one-letter code; pyroglutamic acid derives from glutamine and is therefore coded as pQ).

Confirmation of the sequence

Both pQDPFLRFamide and its Asn^2-analog, pQNPFLRFamide, were synthesized and compared to the natural peptide chromatographically and biologically. The con-

centration of the natural peptide was determined by amino acid analysis. Synthetic pQDPFLRFamide and the natural peptide co-eluted in the same isocratic HPLC solvent system that had been used for the original purification; they were particularly well separated from the Asn^2-analog, as well as from FMRFamide (Fig. 2). The same two peptides also co-eluted in the two gradient elution systems tested and were again separated from the Asn^2-analog. Finally, synthetic pQDPFLRFamide had a higher Rf value (.88) than FMRFamide (.77) on TLC, substantiating an earlier value (.90) found for the ninhydrin-negative, Sakaguchi-positive peptide of *Helix* (Price, 1982).

The natural *Helix* peptide, synthetic pQDPFLRFamide, and FMRFamide are all roughly equipotent on the radula protractor muscle of the whelk, *Busycon contrarium* (Fig. 3). On the heart of *Helix aspersa,* the natural peptide and synthetic pQDPFLRFamide were again equipotent, but in this case, both were about 100 times more active than FMRFamide (Fig. 3). In contrast to their effects on the *Helix* heart, both synthetic and natural pQDPFLRFamide were about 10 times less potent than FMRFamide on the heart of the clam, *Mercenaria mercenaria* (not shown). The action of the Asn^2-analog was not distinguishable from that of pQDPFLRFamide on either the radula protractor or the *Helix* heart, but on the heart of *Mercenaria* this analog was equiactive with FMRFamide and thus distinct from pQDPFLRFamide. In summary, synthetic pQDPFLRFamide and the natural peptide were equipotent in three assay systems, so the proposed sequence is confirmed.

Comparisons of immunoreactivity

pQDPFLRFamide (natural or synthetic) is approximately equipotent to FMRFamide in our RIA (range 80–200%). Furthermore, the remaining, major, unidentified peak in *Helix,* which elutes between FMRFamide and pQDPFLRFamide on HPLC, is also roughly equipotent to FMRFamide. This means that the profiles of immunoreactivity as shown in Figure 1 reflect the actual levels of peptide in the major peaks, and that the total FMRFamide-like immunoreactivity, measured, for example, in the blood, is the unweighted sum of these three major components. The equivalent potency of these three peptides is a peculiarity of our antiserum which reacts more weakly with FLRFamide than FMRFamide, but more strongly with peptides longer than the tetrapeptide; apparently, these two effects are in approximate balance for pQDPFLRFamide. We have tested several antisera raised to conjugates to FMRFamide and find that they, too, react only weakly with FLRFamide (less than 5%); but these antisera have no preference for longer peptides and so react about equally (and weakly) with FLRFamide and pQDPFLRFamide.

Blood immunoreactivity

FMRFamide-like immunoreactivity rapidly disappears if whole *Helix* blood is allowed to stand, even on ice, yet immunoreactive FMRFamide is readily demonstrated if precautions are taken to prevent its enzymatic destruction. We used dilution into acetone to inactivate the enzymes. To see which peptides account for this immunoreactivity, we subjected an extract of about 15 ml of blood to HPLC. The pattern of peaks was the same as that seen with ganglion extracts. The peak that eluted at the expected elution time of pQDPFLRFamide contained 107 pmole, equivalent to a concentration of 7 n*M* in the original blood or about one third of the total immunoreactivity.

We measured the level of immunoreactive FMRFamide in blood from twenty-eight individual snails in various states of activity; the values ranged from 2.58 to 56.85 nanomolar with a mean of 9.5 nanomolar. We could find no significant differ-

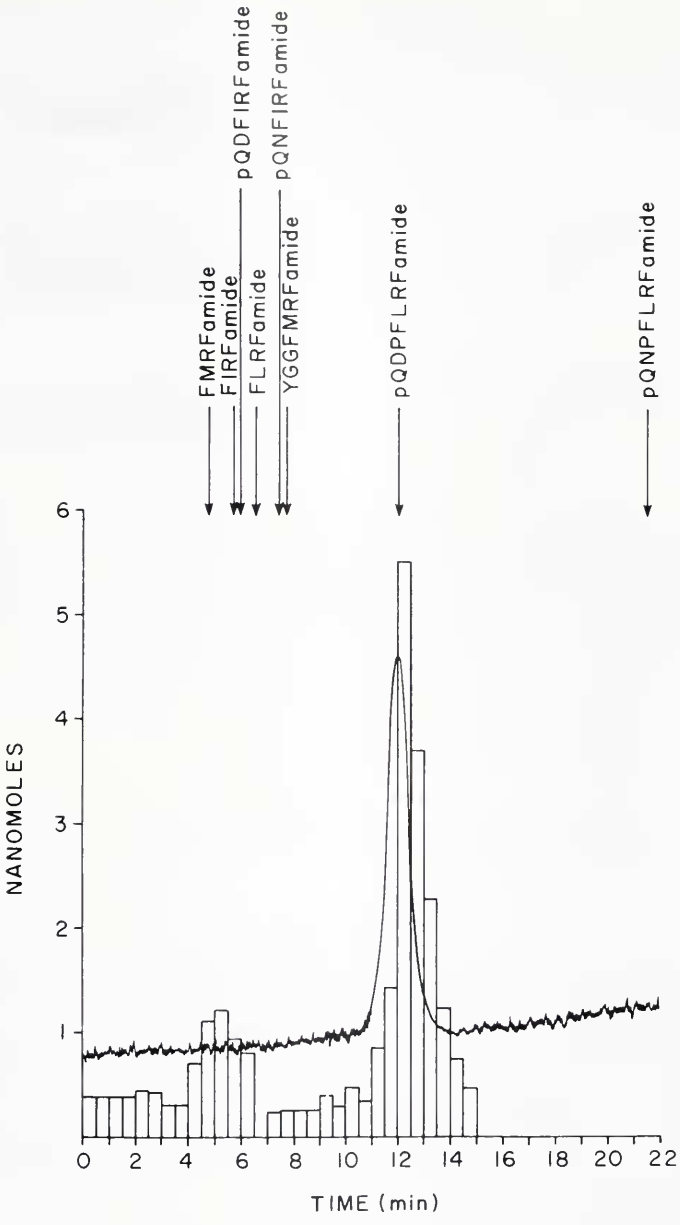

FIGURE 2. A comparison of purified and synthetic FMRFamide-like peptide from *Helix aspersa* (pQDPFLRFamide) by HPLC. Two runs were made and superimposed. In the first, the trace of ultraviolet absorbance at 257 nm was obtained for 100 nmol of synthetic peptide and is about .005 AU at the peak. In the second run, the distribution of natural peptide in the fractions was determined by radioimmunoassay (RIA) and is expressed in terms of the equivalent quantity of FMRFamide (pmol/fraction); about 180 pmol (by amino acid analysis) in 5 μl was injected onto the column (Waters Radial-Pak Micro-Bondapak C-18) which was eluted (4 ml/min) with an ammonium acetate buffer/n-butanol mixture (0.1 *M* acetic acid and 0.5 *M* ammonium acetate; butanol at 80% of saturation); 2 ml fractions were collected.

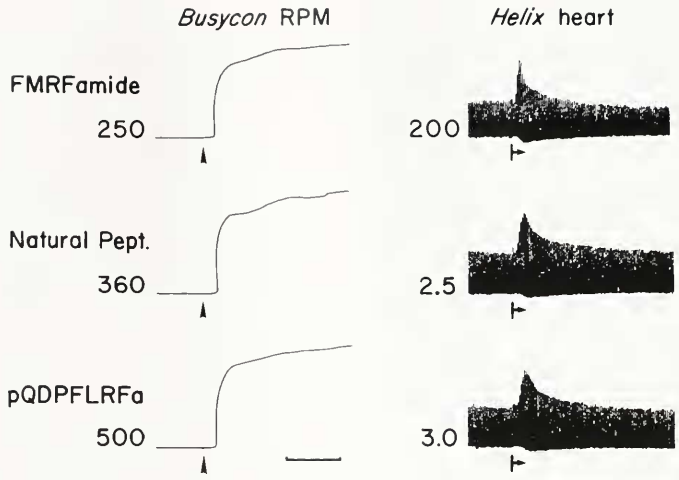

FIGURE 3. Parallel bioassay of FMRFamide and the FMRFamide-like peptide of *Helix aspersa* (pQDPFLRFamide)—both natural and synthetic—on the isolated radula protractor muscle (RPM) of *Busycon contrarium* and the ventricle of *Helix*. The records are of mechanical activity, detected with a force transducer and displayed on an inkwriting oscillograph. Doses: picomoles. The *Busycon* muscles were suspended in aerated seawater (5 ml), and the indicated doses of peptide were added. The *Helix* ventricles were suspended in, and perfused with, saline (Lloyd, 1978), and the indicated doses of peptide were dissolved in saline and injected into the perfusion stream, as follows: synthetic pQDPFLRFamide (300 μl of 10^{-8} *M*); natural peptide (in 200 μl); FMRFamide (200 μl of 10^{-6} *M*). The responses shown, for both the radula protractor and the ventricle, were chosen to be well above threshold and of equivalent height. Since the *Helix* peptide is more slowly acting than FRMFamide, the responses were matched at the point of maximum amplitude of contracture or beat. Time: 1 min.

ences between the levels in active snails and estivating snails, a possible result of our crude sampling method.

DISCUSSION

pQDPFLRFamide is only the first new analog of FMRFamide to be isolated from a mollusc and to be completely characterized; certainly others occur. For example, several peaks of immunoreactivity are evident in extracts of *Helix* ganglia (*e.g.*, Fig. 1B); and there now appear to be at least three distinct, fully processed FMRFamide-like peptides in this snail: pQDPFLRFamide, FMRFamide, and another N-terminally extended analog of FLRFamide (reviewed by Price, 1986). We conclude that *Helix* contains a family of FMRFamide-related peptides. It is now important to determine whether the members of this family occur in different neuronal populations and to compare their biological actions.

Some of the pharmacological actions of pQDPFLRFamide on tissues of *Helix* are markedly different from those of FMRFamide. For example, pQDPFLRFamide is about 100 times more potent on the heart. Also, whereas FMRFamide contracts the retractor muscles of the pharynx and tentacles, the heptapeptide relaxes them (H. K. Lehman and M. J. Greenberg, in prep.). These findings suggest that there are, in *Helix*, multiple receptor sites complementary to the various FMRFamide-like peptides. This notion had previously been proposed on the basis that analogues of FMRFamide could induce, selectively, opposing ionic currents in neurones of *Helix* (Cottrell, 1982).

The pharmacological differences between pQDPFLRFamide and FMRFamide

should have a structural basis. The structure of pQDPFLRFamide differs from that of FMRFamide in two ways: it is extended at the N-terminal, and it contains a leucyl residue in place of the methionyl. Since FLRFamide and FMRFamide are equipotent in several bioassays, we suppose that the presence of leucine instead of methionine does not markedly effect the biological actions of pQDPFLRFamide. Therefore, the N-terminal extention must be critical for the recognition of this peptide by some receptor sites and hence responsible for those differences in pharmacological action that distinguish pQDPFLRFamide fom FMRFamide. This notion is supported by differences in the actions of FMRFamide and t-BOC-FMRFamide on the heart of *Helix* (Greenberg and Price, 1980), and between YGGFMRFamide and FMRFamide on *Helix* neurons (Cottrell, 1982) and on the tentacle retractor muscle of *Helix* (Cottrell *et al.*, 1983a).

Another, less specific effect of the pQDP "tail" of pQDPFLRFamide might be to protect the active FLRFamide sequence from aminopeptidase degradation. In fact, the partial sequence pQXP is found in several unrelated, biologically active peptides including TRF, gastrin, sauvigine, ranatensin, and the hydra head-activator. Thus, this N-terminal sequence may be a convergent stabilizing mechanism. An increased resistance to proteolytic degradation might explain, at least in part, the relatively high potency of pQDPFLRFamide, compared with FMRFamide, on the heart of *Helix*. A more significant consequence of its stability could be that pQDPFLRFamide acts at long range, as a hormone; likely targets in *Helix* would be the heart and the retractor muscles of the pharynx and tentacles. The tentacle muscle is known to be innervated by a FMRFamidergic neuron—C3 from the cerebral ganglion (Cottrell *et al.*, 1983b)—so in this case pQDPFLRFamide (which relaxes the muscle) may be a blood-borne antagonist of FMRFamide.

We have verified, by HPLC and RIA, that pQDPFLRFamide occurs in the blood of *Helix* and accounts for about one-third of the total FMRFamide-like immunoreactivity measured there. The mean blood concentration of pQDPFLRFamide is 3–7 n*M*, and the value in individual snails varies from 1 to 19 n*M*. These are relatively high levels of peptide, and they suggest that pQDPFLRFamide is functioning as a hormone. The targets of this hormone are presently unknown, but a likely one is the heart of *Helix* which is especially sensitive to pQDPFLRFamide. In fact, the threshold for the heptapeptide on the *in vitro* heart ranges between 1 and 10 n*M*, clearly overlapping the range of blood concentrations observed in individual snails. We conclude, therefore, that one hormonal role of pQDPFLRFamide is as a cardioregulator.

The individual variation in the blood levels of FMRFamide-like immunoreactivity may, in part, reflect changes in arousal state caused by our sampling procedure, but the concentration of a functional hormone should, in any event, vary widely with the physiological state of the animal. We must now inquire as to the environmental and physiological variables affecting the levels of peptide in the blood.

ACKNOWLEDGMENTS

This work was supported by NIH grant HL28440 to M.J.G. and is Contribution No. 234 from the Tallahassee, Sopchoppy & Gulf Coast Marine Biological Association. G.A.C. wishes to thank the MRC and Carnegie Trust for financial support. We thank Dr. R. A. Koch for supplying thousands of snails from the gardens of Fullerton, California, and Dr. W. Ross Ellington who collected *Busycon* in the Gulf and put them on the bus to St. Augustine for us. The assistance of Louise MacDonald and Lynn Milstead in preparing this paper is gratefully acknowledged.

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